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Cryopreservation of *In Vitro* Grown Fern Gametophytes

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ABSTRACT.—Two methods of protecting fern gametophyte tissues through exposure to liquid nitrogen (LN) were examined. *In vitro* grown gametophytic tissues from six fern species were exposed to LN after open drying or after encapsulation dehydration, with and without preculture on abscisic acid (ABA). Open drying itself decreased survival with little further effect from LN exposure, although survival was somewhat improved by preculture on ABA. In contrast, encapsulated tissues survived drying and LN exposure at rates comparable to controls (86–100%) irrespective of ABA preculture. Sucrose pretreatment of the encapsulated tissues was important for their subsequent survival through these procedures. Tissues prepared by encapsulation dehydration were successfully regrown after 3.5 years in LN storage. Thus, cryopreservation appears to be a technique which could be used for the stable preservation of *in vitro* cultures of fern gametophytes and for the long-term storage of rare or endangered germplasm of ferns.

Although the more prominent sporophyte and its spores are frequently the focus of study, the small, fragile thallus of the fern gametophyte has also been the subject of much research (Stokey, 1940; Miller, 1968; Dyer, 1979). Fern gametophytes are easily grown in culture, making them good candidates for physiological and developmental studies, but maintaining stock lines or lines from a number of species requires a consistent input of time and labor.

Cryopreservation, or storage in liquid nitrogen (LN) at −196°C, has been used to preserve a variety of living tissues of both vascular and nonvascular plants. Seeds, shoot tips, cell cultures, callus, protoplasts, pollen, and embryos of seed plants (Kartha and Engelmann, 1994; Stanwood, 1985; Pence, 1991), as well as fern spores and the gametophytes of bryophytes (Christianson, 1998; Pence, 1998; and Pence, submitted) have all been successfully maintained in LN.

In this study, the possibility of using cryopreservation to preserve fern gametophytic tissue was explored. Two protocols, which have been used successfully with a variety of other tissues, were tested for preparing gametophyte tissue for cryopreservation: open drying of the tissues before LN exposure and the encapsulation dehydration procedure of Fabre and Dereuddre (1990).

MATERIALS AND METHODS

Gametophyte cultures were initiated from spores of *Davallia fejeensis* Hook., *Drynaria quercifolia* (L.) John Sm., *Cibotium glaucum* (Sm.) Hook. & Arn., *Adiantum trapeziforme* L., *Adiantum tenerum* Swartz., and *Polypodium aureum* L. collected from fronds supplied by Mr. Jeff Kapella, Supervising Florist of the Krohn Conservatory (Cincinnati).

For surface sterilization, spores were wrapped in small packages made by

folding pieces of Whatman No. 1 filter paper. The spores and packages were immersed in a 1:20 dilution of commercial sodium hypochlorite for 5 min, followed by two rinses in sterile distilled water. The packages were then opened, and the spores were blotted onto sterile germination medium, consisting of half-strength Linsmaier and Skoog (LS) (1965) salts and organics, with 1.5% sucrose and 0.22% Phytagel (Sigma Chemical Co.), in 60 × 15 mm disposable plastic petri dishes, approximately 15 ml/dish. The spores were incubated at 26°C under CoolWhite fluorescent lights in a 16/8 hr light/dark cycle. Once germination occurred and gametophytes were formed, the cultures were maintained by subculturing the tissue every 2–3 months onto fresh medium. In some experiments, gametophytes were precultured for one week on this same medium, with and without 10 μM abscisic acid (ABA), which was added to the medium after autoclaving.

For open drying, tissues were cut into pieces, approximately 2–5 mm long, blotted onto sterile filter paper to remove excess moisture and placed in a sterile petri dish under the air flow of the laminar flow hood for 3 hrs.

For encapsulation dehydration, the method of Fabre and Dereuddre (1990) was followed. Tissues were cut into small pieces, approximately 2–3 mm long, and transferred to a solution of 3% alginic acid in calcium-free MS medium plus 0.75 M sucrose. This solution, containing one or more pieces of gametophyte tissue, was then pipeted dropwise into a solution of 100 mM $CaCl_2$, which caused the alginic acid to gel, encasing the tissue in an alginate bead. After 20 min, the beads were removed from the calcium solution and transferred to liquid MS medium containing 0.75 M sucrose, 25 ml in 125 ml flasks, and placed onto a gyratory shaker, 125 rpm, for 18 hr as a pretreatment. In one experiment, different concentrations of sucrose, ranging from 0–30% were tested in the pretreatment step. The pretreated beads were then blotted on sterile filter paper to remove excess moisture and placed on dry filter paper in sterile petri dishes under the air flow of the laminar flow hood to dry for 3–4 hours.

Open dried tissues and dry encapsulated tissues were then placed into sterile 2 ml polypropylene cryovials and immersed directly into LN where they were left either for 1 hr or overnight (no difference was observed between these two LN exposure times). Tissues were thawed by placing the cryovials on the benchtop at ambient temperature for 20 min, after which the tissues or beads were removed and placed onto growth medium for rehydration and recovery. Survival was measured as the recovery of growth from each tissue piece for open dried tissue or the number of beads containing tissues resuming growth. As controls, some tissues were transferred to recovery medium after drying but without LN exposure. With the encapsulation dehydration procedure, tissues which had been pretreated for 18 hours in .75 M sucrose but which had not been dried were cultured as an additional control.

Fern gametophyte tissue from each species was also prepared by the encapsulation dehydration method for long-term cryostorage and banked in LN. After 3.5 years, samples of each were removed from storage and placed onto medium for rehydration and recovery growth.

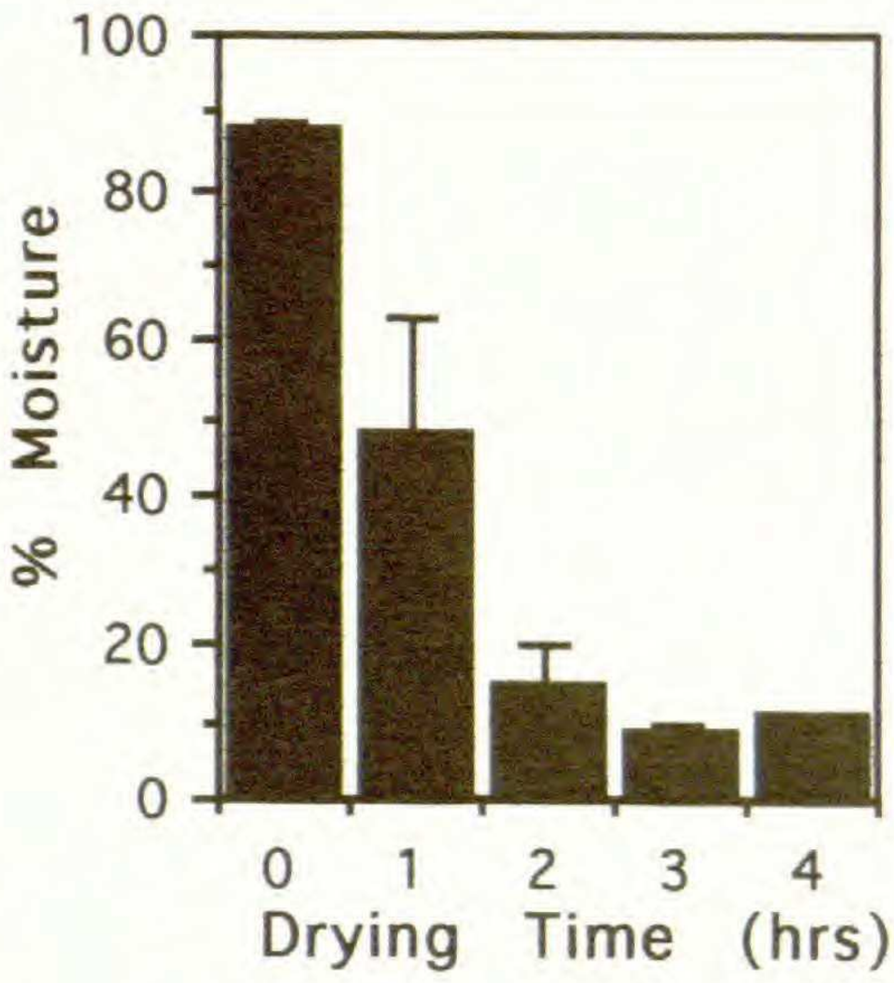

FIG. 1. Moisture loss from open dried gametophytic tissue of *D. fejeensis* during a 4 hour drying period (n = 3).

Moisture determinations were made on tissues of *D. fejeense* harvested at various times during drying under the laminar flow hood. Three samples of groups of small tissue pieces weighing between 0.15 and 0.25 g before drying were used. The percent moisture was calculated on a wet weight basis from the weights of the tissues before and after drying overnight in an oven at 95°C. Samples of tissues encapsulated in alginate beads were also analyzed for moisture after drying in the laminar flow hood.

RESULTS

The moisture level of gametophytes of *D. fejeense* during open drying was reduced to approximately 10% within 3 hours (Figure 1). The gametophytes of the other five species appeared to dry similarly from visual and tactile examination. When tissues and their surrounding alginate beads were dried, moisture levels were somewhat higher, decreasing to only 19–27% during the 3–4 hr drying period.

Survival of gametophyte tissue was reduced after open drying, compared with non-dried controls (Table 1). There was no further decrease in survival with exposure of the dried tissues to LN. *A. trapeziforme, P. aureum* and *C. glaucum* were particularly sensitive to drying, while the other species showed some survival of dried tissues. Preculture on ABA improved survival of the gametophytes through both drying and LN exposure. However, in only one species, *A. tenerum,* was survival of ABA treated tissues equivalent to that of the undried controls.

In contrast, there was excellent survival when gametophytes were encapsulated, pretreated with sucrose and dehydrated in alginate beads prior to LN exposure (Table 2). Tissues showed 100% survival through the 18 hr sucrose pretreatment, with no decrease in viability when the encapsulated tissues were

TABLE 1. Percentage survival and growth of gametophyte tissue pieces of six fern species through 3 hours of open drying followed by LN exposure, with and without preculture on medium containing 10 μM ABA. (n = 8 for controls; n = 10–32 for dried and LN exposed).

Species	Preculture on ABA	% Survival[a]		
		Control	Dried	LN exposed
C. glaucum	−	100	0	10
	+	100	45	88
A. tenerum	−	100	20	60
	+	100	100	100
D. quercifolia	−	100	23	62
	+	100	88	71
D. fejeensis	−	100	58	50
	+	100	92	83
	−	nd	18	30
	+	nd	35	69
P. aureum	−	100	5	0
	+	100	47	33
	−	nd	0	0
	+	nd	6	0
A. trapeziforme	−	100	0	16
	+	100	67	69
	−	nd	0	0
	+	nd	33	39

[a] nd = not determined.

dried. In a few cases, there was a slight decline in survival after LN exposure, but this effect was small (<15%). Because of the high survival rate without ABA, there was no apparent effect of the ABA preculture on survival when encapsulation was used.

Although survival of encapsulated material was good, some damage of the tissues was still evident. Whereas pretreated controls remained consistently green when placed on recovery medium, tissues which were dried or dried and exposed to LN often had some areas which were brownish green in color. Survival came from areas which remained bright green and which eventually grew out and reestablished the culture.

When encapsulated tissues of *D. fejeense* were exposed to different concentrations of sucrose during the 18 hr pretreatment, there was little or no survival through drying and LN exposure when sucrose was omitted completely from the pretreatment medium (Figure 2). However, good survival was observed at all but the highest sucrose concentrations.

Samples of encapsulated gametophyte tissues from these six species showed good survival after 3.5 years in LN storage (Table 3; Figure 3). Survival rates ranged from 50–100%, depending on the species.

DISCUSSION

These results indicate that the encapsulation dehydration procedure can be used successfully to cryopreserve gametophytes of at least six fern species and

TABLE 2. Percentage survival and growth of tissue pieces of six fern gametophytes through encapsulation pretreatment, drying and LN exposure. (n = 5–17). Survival through encapsulation without pretreatment was 100%.

Species	Preculture on ABA	% Survival[a] Sucrose pretrmt	Dried	LN exposed
C. glaucum	−	100	100	100
	+	100	100	100
A. tenerum	−	100	100	94
	−	100	100	100
	+	100	100	93
D. quercifolia	−	100	100	100
	−	100	100	100
	+	100	100	100
D. fejeensis	−	100	100	100
	+	100	100	100
	−	100	100	100
	−	100	100	100
	+	100	100	100
P. aureum	−	100	100	86
	+	nd	100	100
	−	80	82	82
	+	100	100	100
A. trapeziforme	−	100	100	100
	+	nd	100	100
	−	100	100	86
	+	100	100	100

[a] nd = not determined.

suggest that this technique might be broadly applicable to the gametophytes of other fern species, as well. Survival of encapsulated dehydrated gametophytic tissues through LN exposure was generally equivalent to that of controls.

The ability of fern gametophytes to survive drying without encapsulation varied with the species and, in many cases, was very poor. This is not surprising, since these tissues are generally adapted to moist conditions in the wild. ABA has been implicated in increasing stress tolerance in a number of systems (Hartung and Davies, 1991). Preculture on ABA improved the survival of dried fern gametophyte tissues somewhat, but in only one of the six species did survival increase to the level of controls. In a similar study with bryophytes, the effects of ABA on improving tolerance to open drying were also variable, depending on the species (Pence, 1998). However, with these clonal cultures, even a low percentage of survival will regenerate the culture, and open drying with ABA preculture could provide a straightforward method for freezing gametophytes of a number of species.

Survival through the encapsulation dehydration procedure, however, provided a higher level of survival of the fern gametophyte tissue. The presence of sucrose was important to the survival of *D. fejeense* through this method, suggesting that it is an important component in the protection afforded by this

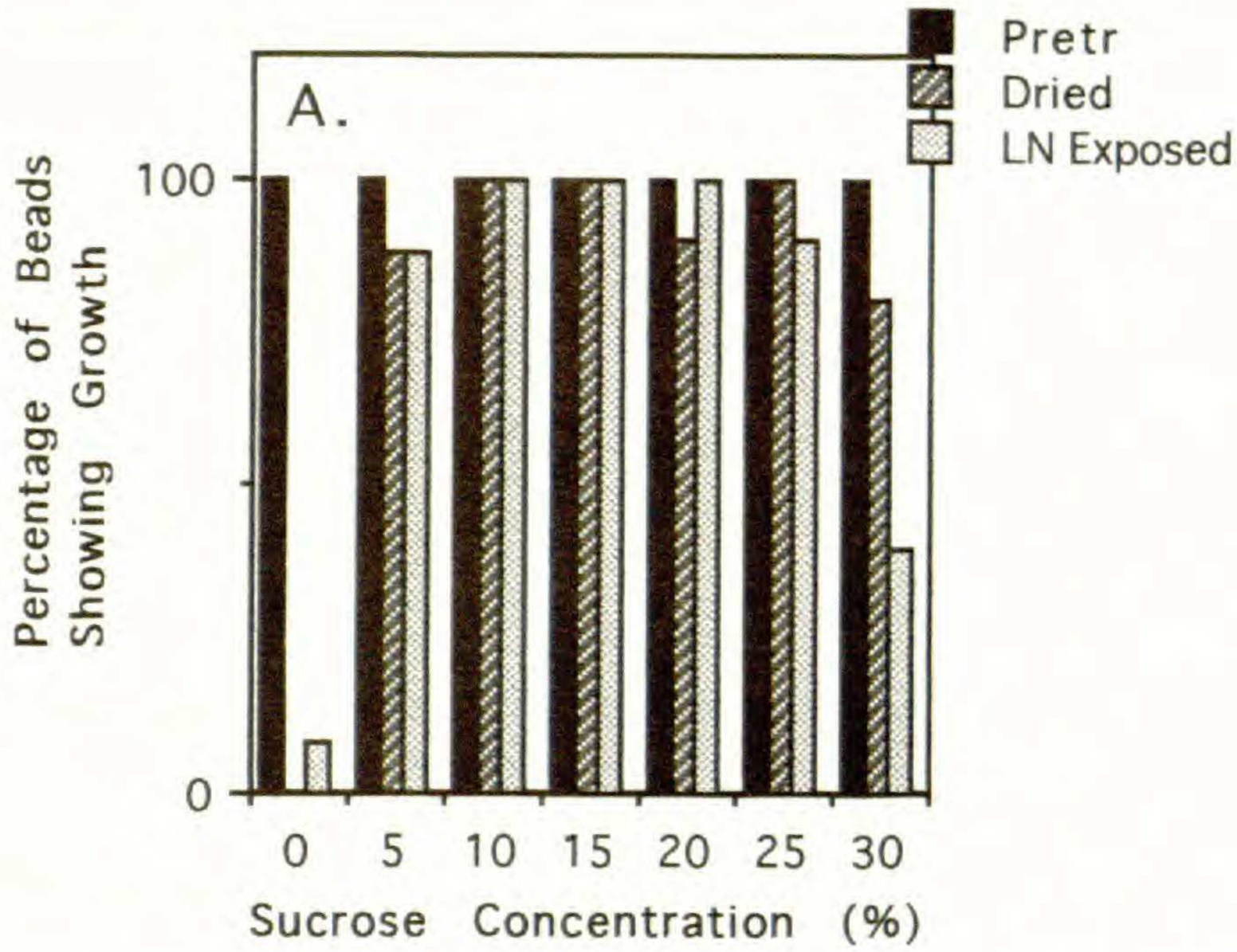

FIG. 2. Growth after drying and LN exposure of gametophytic tissue of *D. fejeensis* pretreated in several concentrations of sucrose using the encapsulation dehydration method (n = 8–10).

procedure. Research in this laboratory with byrophytes has shown that a sucrose pretreatment can improve the survival of open dried tissues (Geiger et al., unpublished). In addition, disaccharides and oligosaccharides have been implicated in the natural desiccation tolerance of several types of plant tissues,

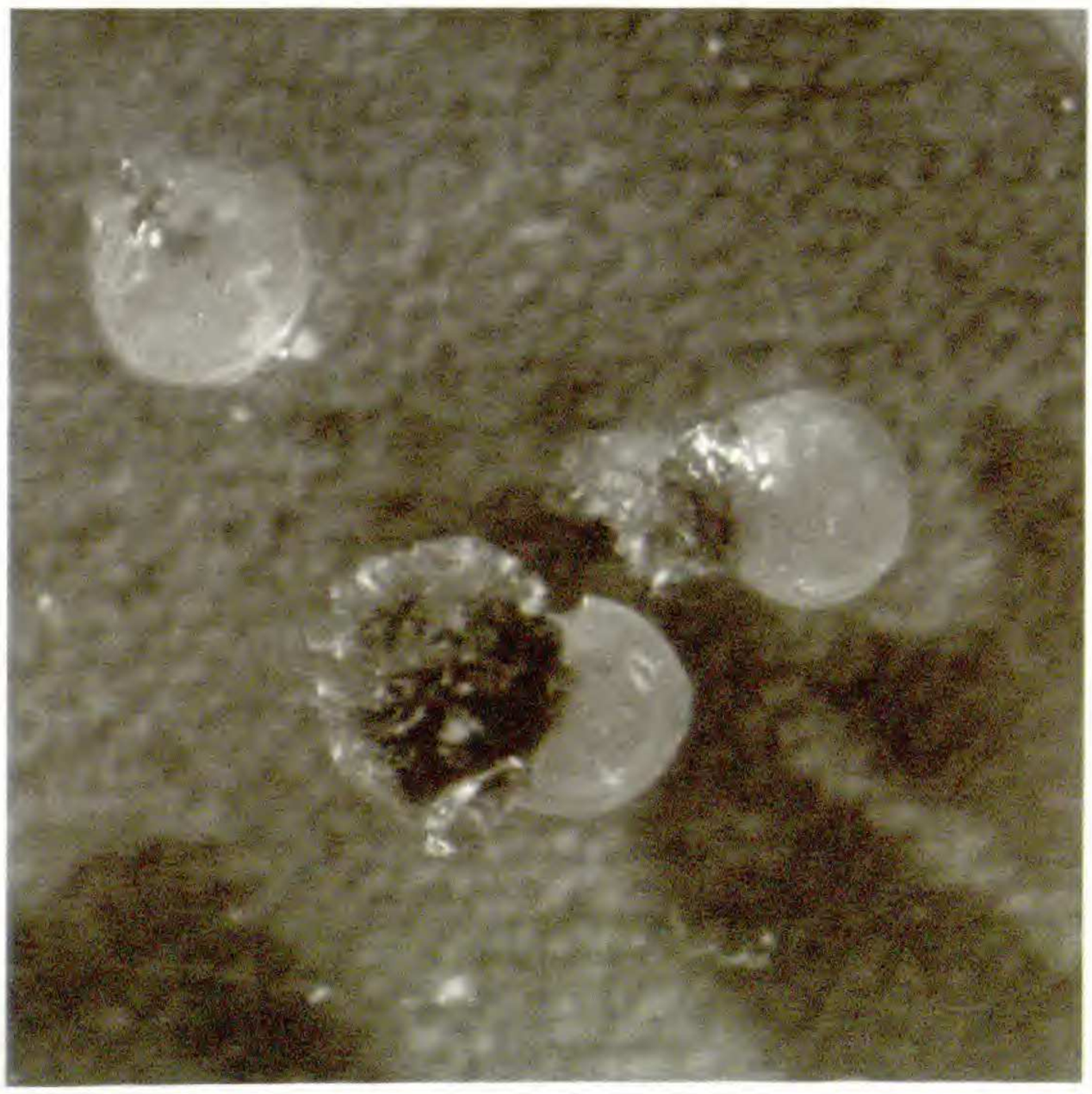

FIG. 3. Gametophyte tissue of *C. glaucum* resuming growth after 3.5 years of storage in LN, prepared for storage with the encapsulation dehydration method. 3.5×

TABLE 3. Regrowth from fern gametophyte pieces which were prepared by the encapsulation dehydration method and stored in LN for 3.5 years.

Species	Number of pieces	Percent of tissue pieces growing
C. glaucum	20	75
A. tenerum	8	88
D. quercifolia	12	67
D. fejeensis	10	100
P. aureum	10	90
A. trapeziforme	6	50

including seeds (Koster and Leopold, 1988), bryophytes (Smirnoff, 1992), and pteridophytes (Adams et al., 1990).

Encapsulation appears to be a technique that should be useful for long-term germplasm storage of fern gametophytes. Tissues resumed growth and appeared normal after 3.5 years of storage in LN, and it is likely that much longer storage times will be achieved. Less than 100% survival was observed with these tissues, in contrast to the experiments done with short-term LN exposure. While the possibility that the longer storage time was detrimental cannot be discounted, it is also possible that other factors, such as the state of the cultures (time since last subculture, etc.), may account for this difference. More tissues will be removed from storage over the next few years in order to determine whether viability does decline with time in storage.

Although survival was less than 100%, each culture was easily regenerated from the stored material. Thus, cryopreservation should be a useful method for preserving research lines and *in vitro* collections of fern gametophyte tissues, decreasing the time and resources necessary to maintain cultures as well as decreasing the opportunity for genetic changes to occur in the cultures.

Gametophytes might also be preserved when spores or sporophytes are not available for germplasm preservation. In the field, some tropical ferns are known only from the gametophyte stage in certain temperate areas (Farrar, 1967), while with other species, gametophytic tissue is more readily available for culture than are spores (Raine and Sheffield, 1997). Studies on soil spore banks and the germination of long-dormant spores can result in gametophytes which may or may not be easily grown into sporophytes (Dyer and Lindsay, 1992; Dyer, 1994). In such situations, cryopreservation could be used for maintaining *in vitro* cultures of such gametophytes for future study.

Nonseed plants have not received as much attention as seed plants with regard to germplasm resources and conservation. However, there are indications that preserving the biodiversity of these organisms could be potentially very important, for example, as sources of useful phytochemicals (Soeder, 1985). Cryopreservation of fern gametophytes is a technique that can supplement *ex situ* spore and sporophyte collections for the long-term storage of fern genetic diversity, providing a stable resource of valuable genetic lines as well as an *ex situ* back-up for species which are rare or endangered in the wild.

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